Supporting Information

Table of Contents

General Remarks

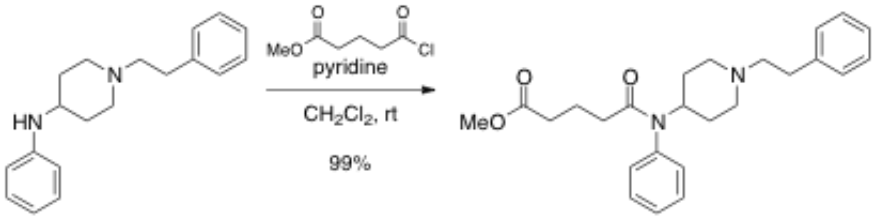
Chemistry

Nuclear magnetic resonance (^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz)) spectra were determined on a Bruker 400 instrument unless otherwise noted. Chemical shifts for ^{1}H NMR are reported in parts per million (ppm) relative to chloroform (7.26 ppm) and coupling constants are in hertz (Hz). The following abbreviations are used for spin multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. Chemical shifts for ^{13}C NMR were reported in ppm relative to the center line of a triplet at 77.0 ppm for chloroform. Electrospray Ionization (ESI) mass were obtained on a ThermoFinnigan LTQ Ion Trap. Matrix-assisted Laser Desorption/Ionization (MALDI) mass spectra were obtained on an Applied Biosystems Voyager DE. Analytical thin layer chromatography (TLC) was performed on Merck precoated analytical plates, 0.25 mm thick, silica gel 60 F_{254}. Preparative TLC (PTLC) separations were performed on Merck analytical plates (0.50 mm thick) precoated with silica gel 60 F_{254}. Flash chromatography separations were performed on Aldrich silica gel (catalog # 717185, 60 Å pore size, 40-63 μm particle size, 230-400 mesh) unless otherwise noted.

Animal Experiments

6-8 week old male Swiss Webster mice (n = 6/group) were obtained from Taconic Farms (Germantown, NY). Mice were group-housed in an AAALAC-accredited vivarium containing temperature- and humidity-controlled rooms, with mice kept on a reverse light cycle (lights on: 9PM-9AM). All experiments were performed during the dark phase, generally between 1PM-4PM. General health was monitored by both the scientists and veterinary staff of Scripps Research Institute, and all studies were performed in compliance with the Scripps Institutional Animal Care and Use Committee, and were in concordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Blood serum samples for titer quantification were performed using tail-tip amputation (<1 cm) in order to collect between 100-150 μL whole blood, and samples then centrifuged at 7500 rpm for 8 min to separate serum.

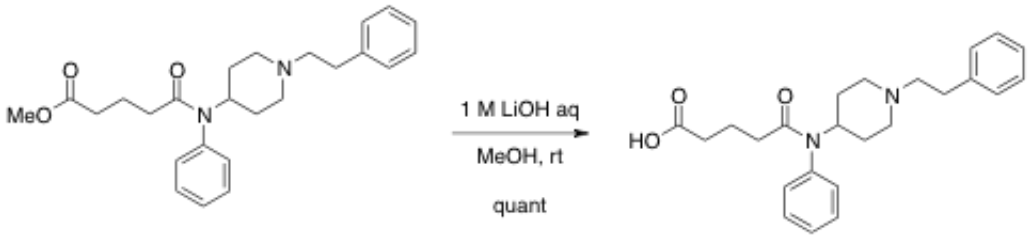
Fentanyl Hapten Synthesis



1 **2**

To a solution of **1**[1] (200 mg, 714 µmol) in CH_2Cl_2 (7.0 mL) were added pyridine (115 µL, 1.42 mmol) and glutaric acid monomethyl ester chloride (110 µL, 780 µmol) at 0 °C. After stirring at 0 °C for 5 min, the reaction mixture was allowed to warm to rt. After stirring at rt for 1.5 h, the reaction mixture was quenched with saturated aqueous $NaHCO_3$, and extracted with EtOAc. The aqueous layer was extracted with EtOAc three times. The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated *in vacuo*. The residual oil was purified by flush column chromatography (SiO_2; *n*-hexane:EtOAc = 1:1 to 1:2 to 0:1) to afford **2** (288 mg, 98.7%) as a pale yellow solid.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.41-7.35 (m, 3H), 7.27-7.23 (m, 2H), 7.19-7.13 (m, 3H), 7.08-7.05 (m, 2H), 4.67 (tt, *J*=12.0, 4.0 Hz, 1H), 3.59 (s, 3H), 2.99 (br d, *J*=12.8 Hz, 2H), 2.72 (dd, *J*=8.4, 8.0 Hz, 1H), 2.72 (d, *J*=6.4 Hz, 1H), 2.53 (dd, *J*=8.4, 7.2 Hz, 1H), 2.26 (t, *J*=7.2 Hz, 2H), 2.15 (td, *J*=12.4, 2.4 Hz, 2H), 1.98-1.93 (m, 2H), 1.87 (qd, *J*=7.6, 1.6 Hz, 3H), 1.85-1.77 (m, 2H), 1.42 (qd, *J*=12.4, 3.6 Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 173.5, 171.7, 139.7, 138.4, 130.2 (2C), 129.3 (2C), 128.5 (2C), 128.3, 128.3 (2C), 126.0, 59.9, 52.6 (2C), 51.9, 51.3, 33.9, 33.2, 33.1, 29.9 (2C), 20.5; HRMS (ESI+) 409.2487 (calcd for $C_{25}H_{33}N_2O_3$ 409.2491).

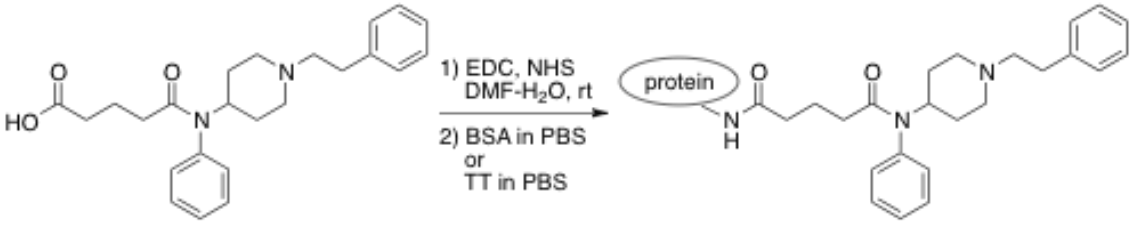


2 **3**

To a solution of **2** (49.9 mg, 122 μmol) in MeOH (1.0 mL) was added 1.0 M aqueous LiOH (250 μL) at rt. After stirring at rt for 4 h, the reaction mixture was washed with hexane. The aqueous layer was acidified with 2.0 M aqueous HCl, and extracted with EtOAc three times. The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated *in vacuo*. The residual white solid **3**[2] (52.0 mg, quant) was used for the next step without further purification. The spectroscopic data for **3** were collected after purification by PTLC (SiO_2; CH_2Cl_2:MeOH = 9:1).

^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.41-7.35 (m, 3H), 7.29-7.24 (m, 2H), 7.22-7.18 (m, 1H), 7.17-7.15 (m, 2H), 7.06-7.04 (m, 2H), 4.83 (br s, 1H), 4.69 (tt, *J*=12.0, 4.0 Hz, 1H), 3.35 (br d, *J*=11.6 Hz, 2H), 2.96-2.85 (m, 4H), 2.56 (br t, *J*=10.8 Hz, 2H), 2.16 (t, *J*=7.2 Hz, 2H), 1.98 (t, *J*=7.2 Hz, 2H), 1.87 (br d, *J*=12.4 Hz, 2H), 1.83-1.74 (m, 2H), 1.80 (t, *J*=6.8 Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 176.9, 172.3, 139.0, 138.4, 130.0 (2C), 129.5 (2C), 128.6 (2C), 128.6, 128.5 (2C), 126.3, 59.2, 52.3 (2C), 51.7, 34.3, 34.1, 32.3, 29.2 (2C), 20.9; HRMS (ESI+) 395.2327 (calcd for $C_{24}H_{31}N_2O_3$ 395.2335).

Fentanyl Hapten Conjugation to Bovine Serum Albumin (BSA) and Tetanus Toxoid (TT)

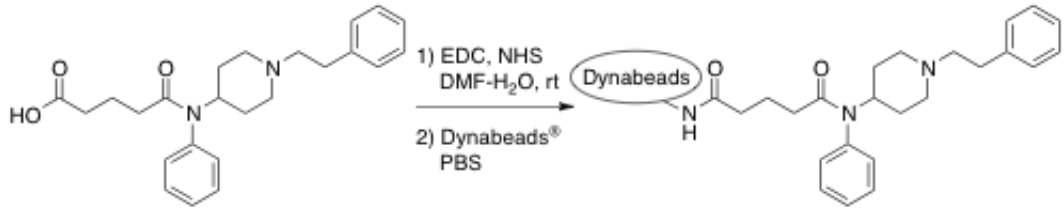


3 **4**

To a solution of **3** (2.7 mg, 6.8 μmol) in DMF (144 μL) and H_2O (16 μL) were added NHS (*N*-hydroxysuccinimide) (4.8 mg, 40.4 μmol) and EDC (*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide) (7.8 mg, 40.8 μmol) at rt. After stirring at rt for 1.5 h, additional EDC (4.2 mg, 21.9 μmol) was added. After additional stirring at rt for 2 h, the reaction mixture was divided into two portions.

One portion (80 μL) was added into a solution of bovine serum albumin (BSA, Thermo Scientific) (660 μL, 1.50 mg/mL) in phosphate-buffered saline (PBS, pH 7.4) at rt, and the other portion (80 μL) was added into a solution of tetanus toxoid (TT, Statens Serum Institut) (650 μL, 1.53 mg/mL) in PBS (pH 7.4) at rt. After stirring at rt for 15 h, each of the reaction mixture was dialyzed against PBS (pH 7.4) at rt using a Slide-A-Lyzer 10 K MWCO dialysis device. The buffer was exchanged every 2 h for 6 h, and then dialysis was continued for 12 h at 4 °C to afford the purified fentanyl-TT conjugate (Fent-TT, **4a**) and the fentanyl-BSA conjugate (Fent-BSA, **4b**). The conjugate concentrations were quantified by BCA assay, and the conjugates were stored at 4 °C.

Preparation of Fentanyl Hapten Coated Dynabeads® M-270 Amine



3 **5**

To a solution of **3** (3.0 mg, 7.6 μmol) in DMF (225 μL) and H_2O (25 μL) were added NHS (9.0 mg, 75.8 μmol) and EDC (14.6 mg, 76.1 μmol) at rt. After stirring at rt for 12 h, a 50 μL aliquot of the reaction mixture was added into 1.0 mL of Dynabeads® M-270 Amine (washed 4 times with PBS (pH 7.4) prior to use) in PBS at rt. After stirring at rt for 1.5 h, the beads were washed with PBS (pH 7.4) for two times and stored at 4°C.

MALDI-TOF Analysis

In order to quantify copy number (hapten density) for each fentanyl hapten-BSA and TT conjugates prepared in this study, samples were submitted for MALDI-TOF analysis and compared MW of fentanyl hapten-BSA and TT conjugates with MW of unmodified BSA and TT, respectively, as per the formula:

$$\text{copy number} = (MW_{\text{fentanyl hapten-protein}} - MW_{\text{protein}}) / (MW_{\text{fentanyl hapten}} - MW_{\text{water}})$$

$$MW_{\text{fentanyl hapten}} = 395 \text{ Da}, MW_{\text{water}} = 18 \text{ Da}$$

$$MW_{\text{fentanyl hapten-BSA}} = 80{,}939 \text{ Da}, MW_{\text{BSA}} = 66{,}500 \text{ Da}$$

$$MW_{\text{fentanyl hapten-TT}} = 168{,}757 \text{ Da}, MW_{\text{TT}} = 153{,}500 \text{ Da}$$

$$\text{copy number}_{\text{fentanyl hapten-BSA}} = 38$$

$$\text{copy number}_{\text{fentanyl hapten-TT}} = 40$$

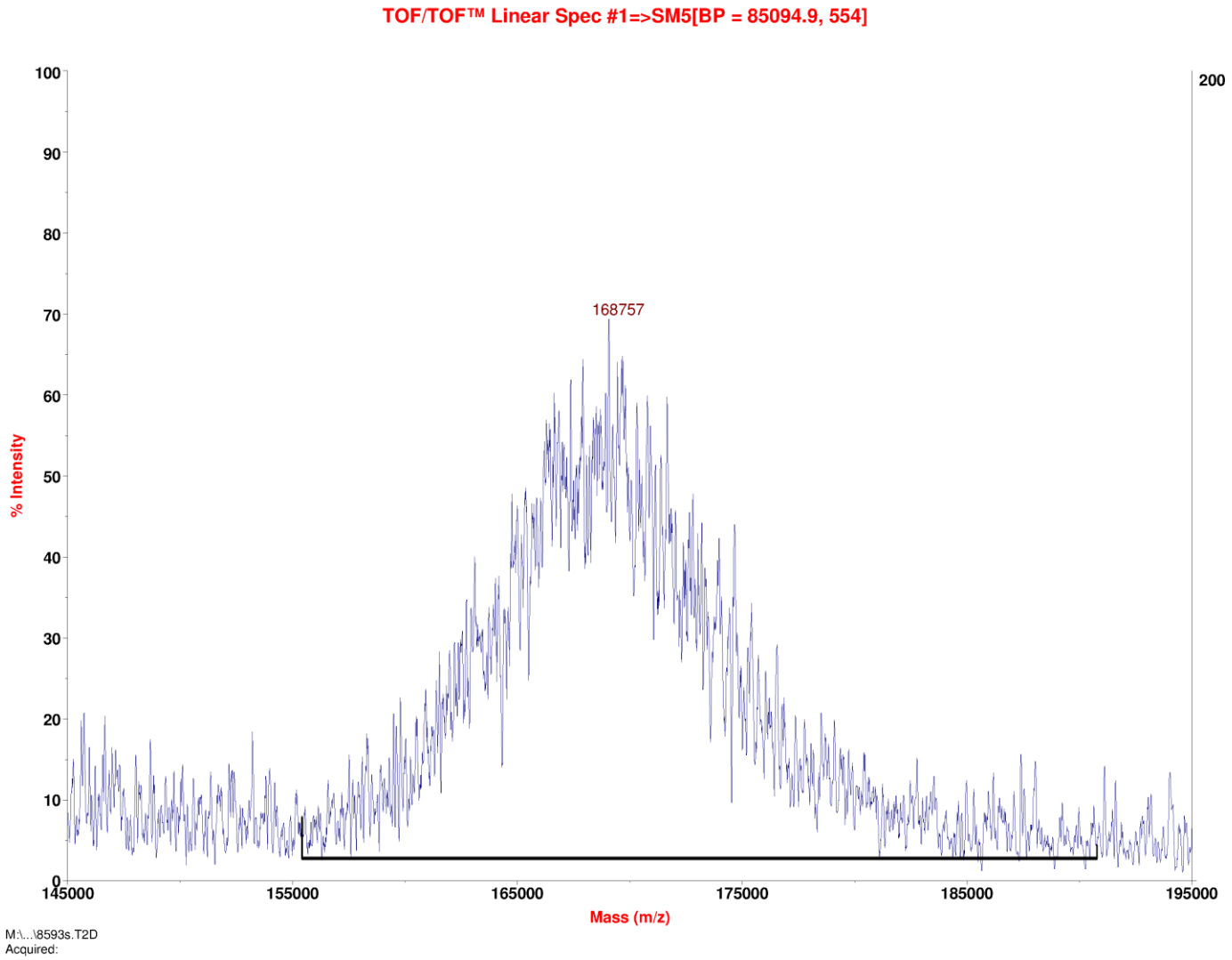


Figure S1a. MALDI-TOF mass spectrum of fentanyl hapten-TT conjugate **4a**.

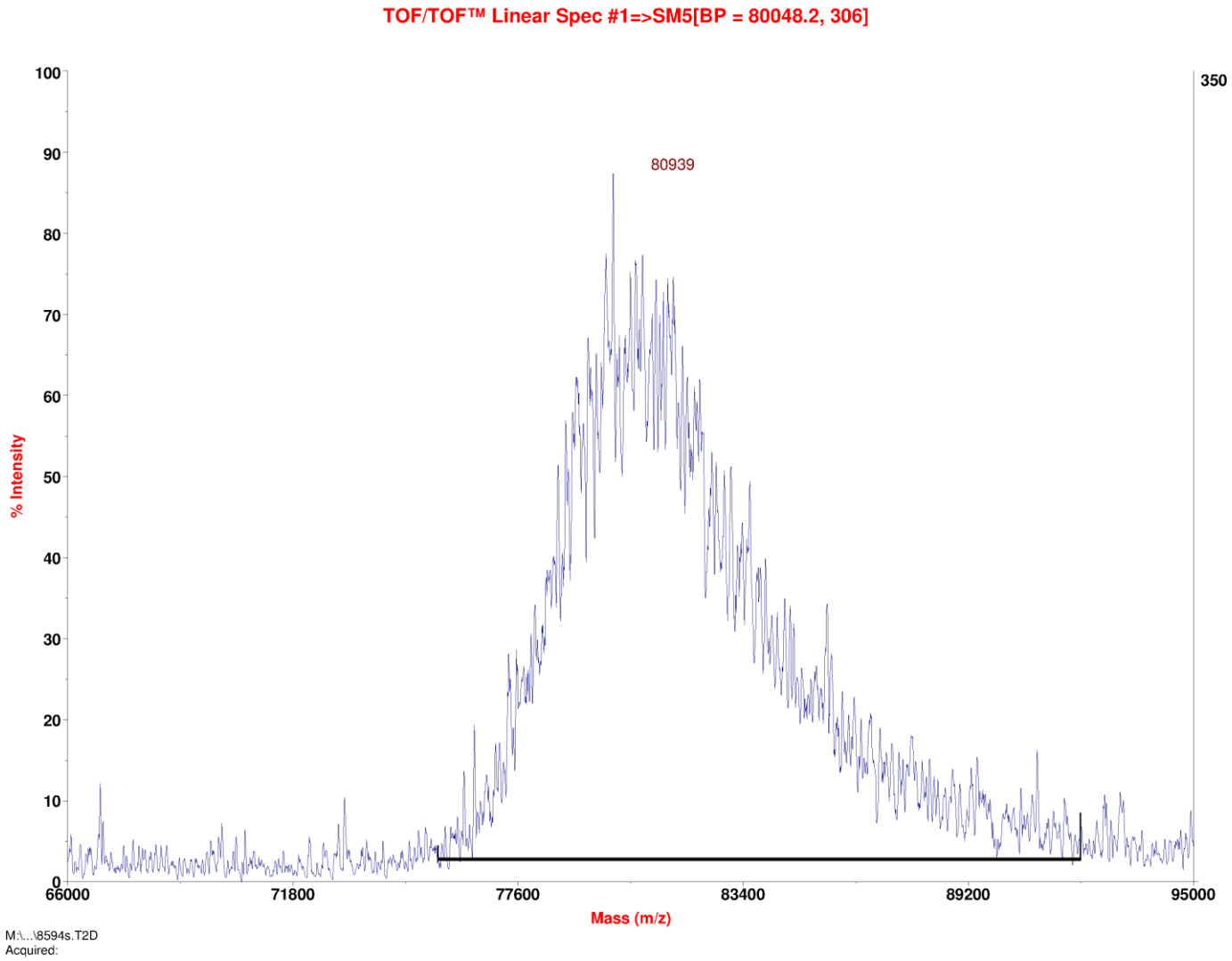


Figure S1b. MALDI-TOF mass spectrum of fentanyl hapten-BSA conjugate **4b**.

ELISA Procedure

Pipetting and washing steps were performed on a Biomek 4000 liquid handling robot. PBS was used throughout the assay at pH 7.4 and was prepared from a 10X powder packet from Fisher Scientific. First, half-area high-binding 96-well microtiter plates (Costar 3690) were coated with 25 µg of Fent-BSA per well overnight at 37 °C, allowing the liquid to evaporate. Following blocking with skim milk for 1 h at rt, vaccinated mouse serum was serially diluted 1:1 in 2% BSA solution across the 12 columns starting at 1:5000. After a 2 h incubation at rt, the plates were washed 5X and donkey anti-mouse IgG horseradish peroxidase (HRP) secondary (Jackson ImmunoResearch) at a 1:10,000 dilution in 2% BSA was added and incubated for 1 h at rt. 5X washing was performed and 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Thermo Pierce) was added, followed by 2M H_2SO_4 5 min after TMB addition. Plates were allowed to incubate 20 min before their absorbances were read at 450 nm. In GraphPad PRISM, absorbance values were normalized to the highest absorbance value per sample, and a curve was fit using the log(inhibitor) vs. normalized response – variable slope equation to determine the midpoint titer and standard errors. Non-vaccinate mice did not contain any detectable anti-fentanyl titers.

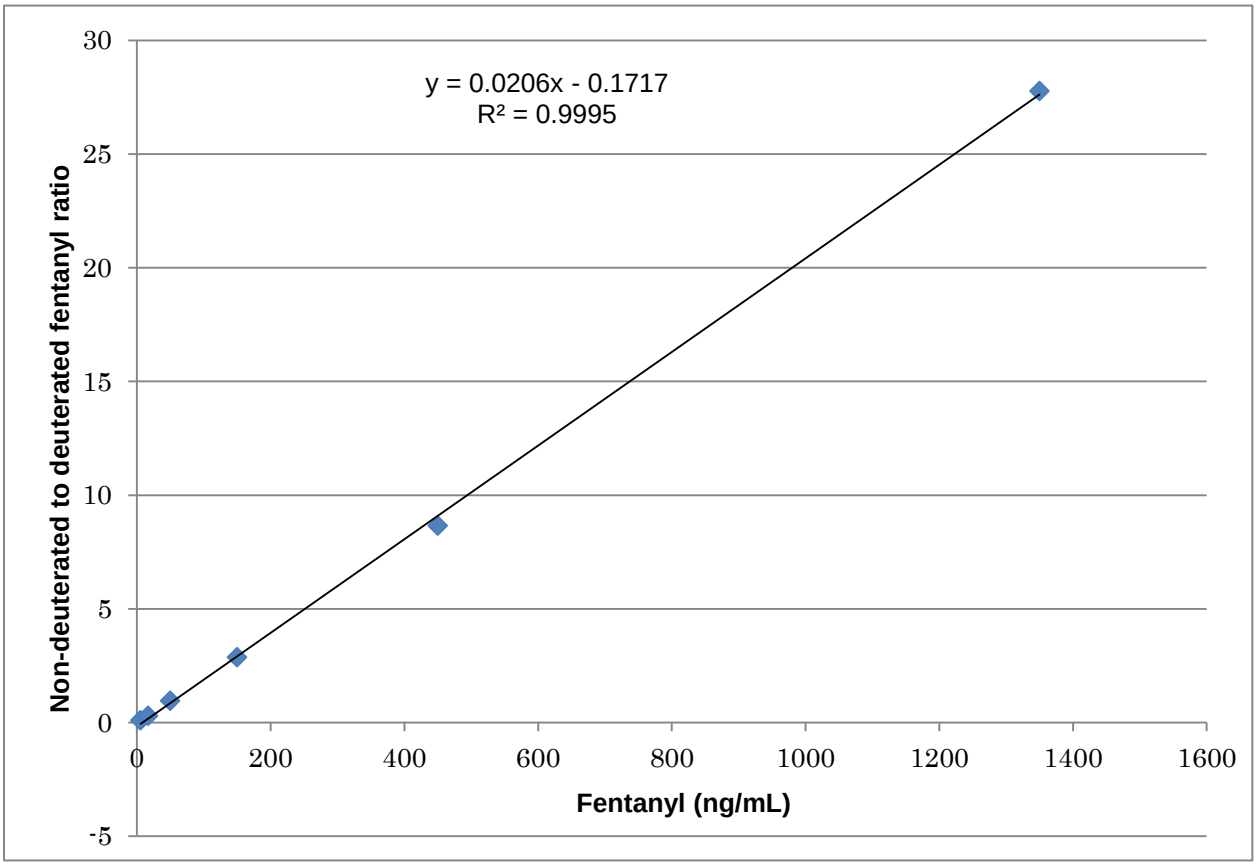
Competitive ELISA was also performed in a similar manner but with an added step: serum at the IC_{80} dilution was incubated with free fentanyl dilutions of 1 mM to 0.1 nM (eleven 5-fold dilutions) in Fent-BSA coated plates for 2 h. The IC_{50} was calculated to be 1.24 ± 0.44 µM, which is off by a factor of about 200 compared to SPR results.

Animal Procedure for Blood/Brain Biodistribution Study

Mice (n=6 vaccinated and n=6 control) were injected subcutaneously with 0.2 mg/kg fentanyl in a 10 mL/kg volume of physiological sterile saline (an established fully analgesic drug dose in naïve mice). 15 min following injection, mice were fully anesthetized using nose cones constructed from 50 mL Falcon® conical centrifuge tubes (Corning, NY) containing gauze pads soaked in isoflurane. Mice were then opened along the midline just below the sternum and the diaphragm peeled back to expose the heart. Cardiac puncture yielded roughly 1.5 mL of whole blood. Immediately following cardiac puncture, mice were rapidly decapitated using large surgical scissors and brain extracted with rongeurs. The brain was then weighed (typically between 4.0-6.0 g) and lightly washed in a 1.0 mL solution of ice-cold standard PBS to remove excess external blood; however, mice were not perfused to fully remove all blood that may have been contained within ventricles and internally within the brain matter. Brains were then added to 1.0 mL fresh ice-cold PBS in 5.0 mL conical sample tubes and homogenized using a Tissue Tearor (Biospec; Bartlesville, OK). Samples of both brain and serum were then frozen until sample prep for LCMS analysis.

Sample Preparation for LC-MS Analysis of Fentanyl in Blood and Brain[3]

To an aliquot of sample (400 µL, blood or homogenized brain) was added fentanyl-d_5 (20 µL, 50 ng/mL) in MeOH as an internal standard, and then the mixture was vortex mixed and allowed to equilibrate. After 30 min for equilibration, H_2O (400 µL) was added to the mixture. Basification was performed by addition of 0.1 M aqueous K_2CO_3 solution (400 µL) followed by agitation using a vortex mixer. Extraction was conducted with mixture of *n*-hexane and EtOAc (7:3) (2.8 mL). After vortex mixing for 2 min and centrifugation at 3000 rpm for 5 min, the organic layer was evaporated using GENEVAC®. An 8 µL aliquot was injected into LC-MS system equipped with an Agilent Poroshell 120 SB-C8 column using 5 mM NH_4OAc pH 4/acetonitrile mobile phase. A blank was injected before every sample. Deuterated and non-deuterated masses were extracted in MassHunter and resulting peaks were integrated. Using the ratio of non-deuterated to deuterated integration values, fentanyl concentrations were determined via a six point standard curve (see below). Standards for the calibration curve were prepared in the same manner as tissue samples except with solutions of known non-deuterated concentrations of fentanyl.



Vaccine Formulation and Administration

On a per mouse basis, 50 µg Fent-TT + 50 µg CpG ODN 1826 (Eurofins) in 75 µL pH 7.4 PBS was combined with 75 µL (0.75 mg) Alhydrogel (Invivogen) and mixed for 30 min. The suspension (150 µL per mouse) was injected intraperitoneally to 6 mice at weeks 0, 2, 4. Mice were bled at weeks 2, 4, 6, 10 and 12.

Opioid Antinociceptive Potency Testing

At least 2 days following a bleed, mice were tested for cumulative fentanyl response in primarily supraspinal (hot plate) and spinal (tail immersion) behavioral tests as previously described.[4] The hot plate test was measured by placing the mouse in an acrylic cylinder (14 cm diameter * 22 cm) on a 54 °C surface and timing latency to perform one of the following nociceptive responses: licking of hindpaw, shaking/withdrawal of hindpaw, or jumping. Typical baseline latency was between 8-15 s and a 35 s cutoff was imposed to prevent tissue damage; after response mice were removed from the plate. The tail immersion test was administered by lightly restraining mice in a small pouch constructed from absorbent laboratory underpads and dipping 1 cm of the tip of the tail into a heated water bath, with the time to withdrawal timed. Typical baseline response was 1-2 s and a cutoff of 10 s was used to prevent tissue damage. Since tail immersion is a more reflexive behavior, testing order was always hot plate first followed by tail immersion. Immediately following completion of both antinociceptive assays, fentanyl (5.0 mL/kg in normal saline) was immediately injected subcutaneously. Testing was repeated roughly 10 min following each injection, and this cycle of testing and injections was repeated with increasing cumulative fentanyl dosing until full antinociception (i.e. cutoff times surpassed) was observed in both assays. Upon completion of all testing, mice were administered a cocktail of 1.0 mg/kg naloxone and naltrexone in saline to prevent subsequent consequences of potential overdose.

Lethality Study

Male Swiss Webster mice (N=11) were injected intraperitoneally with 2.2 mg/kg fentanyl HCl in pH 7.4 PBS after which 2/11 died of overdose. A second 2.2 mg/kg fentanyl HCl dose was administered 15 min later to the remaining 9 mice after which four more (6/11) died of overdose. The same experiment (N=12) was performed subcutaneously with cumulative dosing of 0.4, 0.8, 1.6, 2.4 and 4.4 mg/kg over 1 h inducing overdose deaths in 2/12 of the mice.

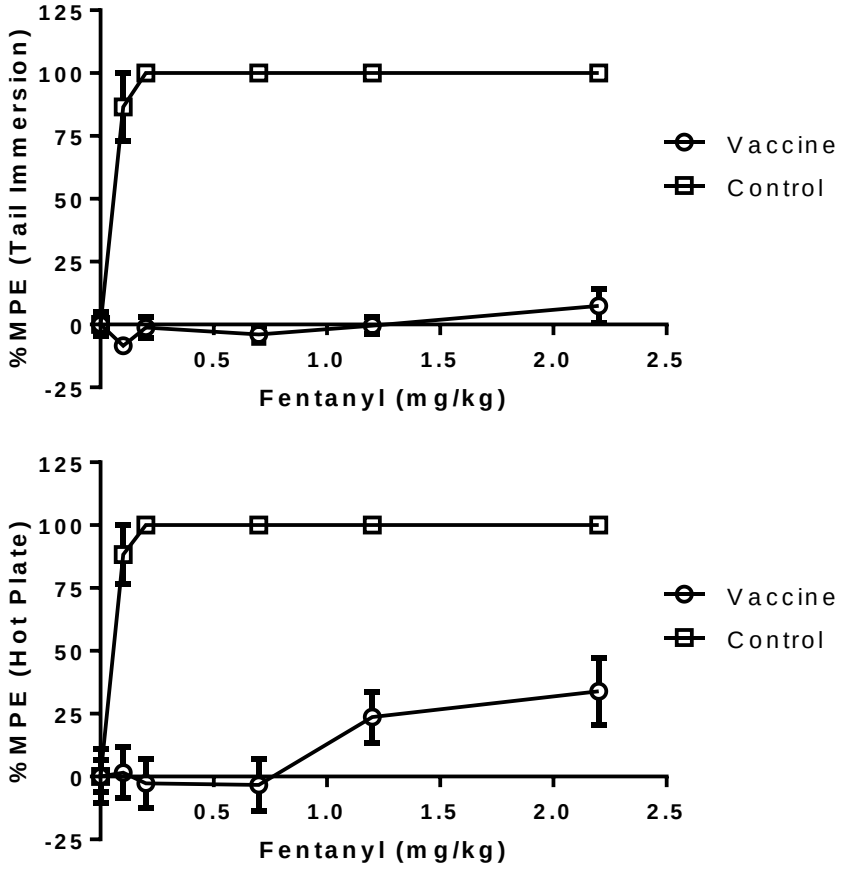


Figure S2. Fentanyl antinociceptive testing of control and vaccine animals (n=6 each) at week 6.

Statistics

Statistical analysis was performed in GraphPad Prism 6 (La Jolla, CA). All values are reported as means ± SEM. Antinociceptive data was transformed from time to % maximum possible effect (%MPE), which is calculated as: %MPE = (test – baseline) / (cutoff – baseline) * 100. This data was then fit using a log(agonist) vs. normalized response non-linear regression. ED_{50} values and 95% confidence intervals of the ED_{50} were calculated for each pain test and individual treatment group to determine potency ratios. An unpaired *t* test was used for verification of statistically significant differences ($\alpha < 0.001$) between control and vaccinated groups for blood/brain fentanyl concentrations and fentanyl ED_{50} shifts.

Determination of Binding IC_{50}s for Mouse anti-Fentanyl Immunoglobulins (IGs)

The binding IC_{50} for mouse IGs and free fentanyl was determined by competitive binding assay via surface plasmon resonance using a Biacore 3000 instrument (GE Healthcare) equipped with a research-grade CM3 sensor chip. The ligand, fentanyl-BSA conjugate, was immobilized using NHS, EDC coupling reaction. The surface of all two flow cells (flow cells 1 and 2) were activated for 7 min with a 1:1 mixture of 0.1 M NHS and 0.1 M EDC at a flow rate of 5 µL/min. The ligand resuspended in 10 mM sodium acetate (pH 4.0) was immobilized at a density of 2,000 RU on flow cell 2; whereas flow cell 2 was immobilized with BSA at the same density to serve as a reference surface. All the surfaces were blocked with a 7 min injection of 1.0 M ethanolamine-HCl (pH 8.5). The mouse IGs were diluted in running buffer (HBS-EP+ buffer) and titrated on both coated flow cells, so as to give a response of ~ 60 RU with 3 min of injection and 2.5 min dissociation at a flow rate of 30 µL/min. The mouse IGs prepared in HBS-EP+ buffer at determined concentration was incubated with a series concentration of compounds for 1 h at room temperature before conducting the competitive binding assay. The compounds and their concentration series are as follows: a) fentanyl, ranging from 10 µM to 169 pM with a three-fold dilution series; b) acetylfentanyl, butyrylfentanyl, and *p*-tolylfentanyl, ranging from 50 mM to 850 pM with three-fold dilution; c) *cis*-3-methylfentanyl, and α-methylfentanyl (China White), ranging from 100 mM to 95 pM, four-fold dilution series; d) methadone and oxycodone, ranging from 100 mM to 10 nM, ten-fold dilution series. Note, all fentanyls were racemic. To collect binding data, the analyte, the mouse IGs and compound mixture, was injected over the two flow cells at a flow rate of 30 µL/min at 25 °C for 3 min and was dissociated in buffer for 2.5 min before regeneration. The chip surface was regenerated by injection of 10 mM Gly-HCl (pH1.5) for 30 seconds before the next round of assay. The response at the end of dissociation phase for each cycle of binding analysis was used to calculate the IC_{50} value for each compound by GraphPad Prism 6 software. The binding curves are illustrated in Figure 5a.

Determination of Binding Kinetics for Purified Mouse anti-Fentanyl Immunoglobulins (IGs)

The binding kinetics between mouse IGs (purified directly from week 6 bleed using magnetic fentanyl-coupled Dynabeads) and free fentanyl were determined by surface plasmon resonance using a BiOptix 404pi instrument (BiOptix Diagnostics, Inc., Boulder, Co.) equipped with a CMD200m sensor chip. The ligand, mouse anti-fentanyl IgGs (~150 kDa), were immobilized using NHS, EDC coupling reaction. The surface of all two flow cells (flow cells 2 and 3, assay set at 2 * 2 injection mode) were activated for 7 min with a 1:1 mixture of 0.1 M NHS and 0.1 M EDC at a flow rate of 5 µL/min. The ligand resuspended in 10 mM sodium acetate (pH 4.5) was immobilized at a density of 12,000 RU on flow cell 3; whereas flow cell 2 was immobilized with a non-related antibody at the same density to serve as a reference surface. All the surfaces were blocked with a 7 min injection of 1.0 M ethanolamine-HCl (pH 8.5). To collect kinetic data, the analyte, fentanyl (336.48 Da) prepared in HBS-EP+ buffer (10 mM HEPES, 150 mM NaCl, 0.05% P20 (pH 7.4)), was injected over the two flow cells at concentration range from 1,000 – 1.95 nM (two-fold dilution series) at a flow rate of 50 µL/min at a temperature of 25 °C. The complex was allowed to associate and dissociate for 300 and 900 s, respectively. Duplicate injections (in random order) of each analyte sample and blank buffer injections were flowed over the two surfaces. Data were collected, double referenced, and were fit to a 1:1 interaction model using the global data analysis by Scrubber 2. The kinetic data are shown in Figure 5b.

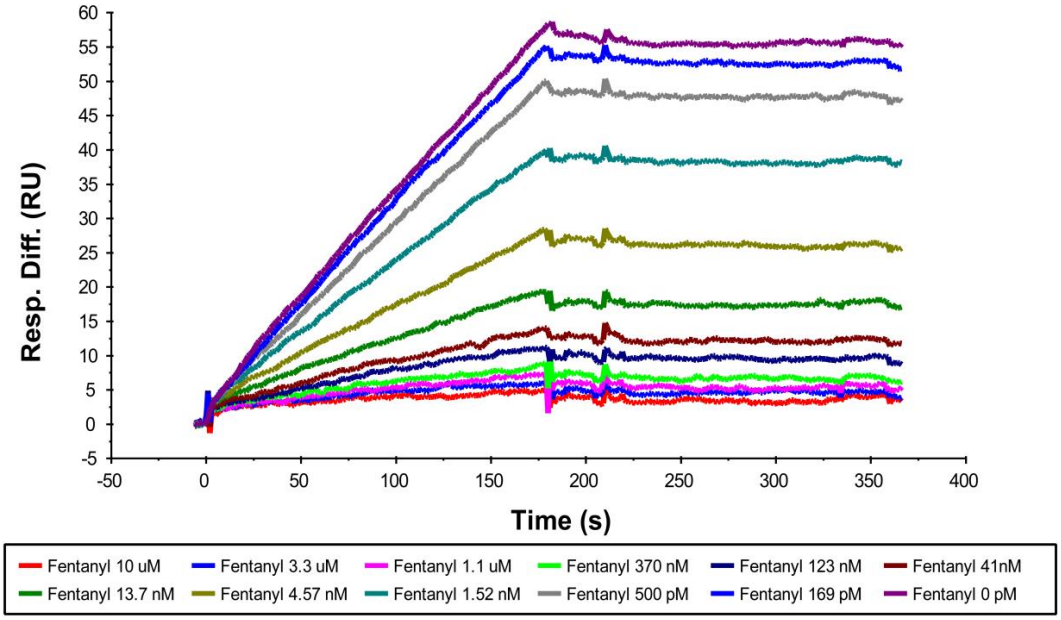


Figure S3. Overlaid plots of sensorgrams obtained at 25 °C for the competitive interaction between anti-fentanyl sera and fentanyl-BSA conjugate with free fentanyl compound as competitor, which are presented at concentrations of 10,000, 3,300, 1,100, 370, 123, 41, 13.7, 4.57, 1.52, 0.5, 0.169 and 0 nM.

References

[1] C. A. Valdez, R. N. Leif, B. P. Mayer, *Plos One* **2014**, *9*.

[2] R. Vardanyan, V. K. Kumirov, G. S. Nichol, P. Davis, E. Liktor-Busa, D. Rankin, E. Varga, T. Vanderah, F. Porreca, J. Lai, V. J. Hruby, *Bioorg Med Chem* **2011**, *19*, 6135-6142.

[3] V. Coopman, J. Cordonnier, K. Pien, D. Van Varenbergh, *Forensic Sci Int* **2007**, *169*, 223-227.

[4] P. T. Bremer, J. E. Schlosburg, J. M. Lively, K. D. Janda, *Mol Pharm* **2014**, *11*, 1075-1080.